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TITLE: Progression of Inflammatory Bowel Disease to Cancer: Is the Patient
"Better Off" without Lymphatic Vessels or Nodes (or Angiopoietin 2)?

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14. ABSTRACT This proposal addresses inflammatory bowel disease (IBD). Multiple factors have been implicated in the progression of ulcerative/granulomatous (Crohn's) colitis to colorectal carcinoma (CRC) in IBD patients and experimental models. Nonetheless, the pathogenic link, interrelationship, and practical clinical application of these various theories of progression have remained elusive. We proposed that a reduced number of functioning lymphatic vessels and impaired lymph drainage (lymphatic vascular insufficiency) in the colon actually protects against progression of inflammatory colitis to CRC. Our primary objective is to determine whether there is a reduced incidence of CRC in mice with lymphatic insufficiency from genetic knockout of angiopoietin2 (Ang2) compared to controls. We have: 1) completed and secured approval of ACURO Appendix; 2) revised dextran sodium sulfate (DSS) dosing in our model; 3) completed mouse cohorts chronically exposed to DSS; 4) added pre-carcinogen azoxymethane (AOM) before chronic DSS; and 5) completed additional experimental groups, and are continuing final data analysis and latest imaging studies. This project has potentially high impact because of the substantial incidence of IBD-CRC progression with associated morbidity and mortality and importance of clarifying positive and negative interactions of lymphatic functional status and Ang2, identifying potential biomarkers, and developing new imaging, preventive, and treatment approaches to IBD-induced CRC.					
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**Interim Annual Report
DOD #W81XWH-II-I-0727 30/09/2011-24/2/14
PI Marlys H. Witte, MD, Professor of Surgery
University of Arizona**

Introduction

This Concept project has addressed inflammatory bowel disease (IBD) and progression to colorectal cancer (CRC). Multiple factors have been implicated in the progression of ulcerative/granulomatous (Crohn's) colitis to CRC in patients and in experimental models of IBD. Nonetheless, the pathogenic link, interrelationship, and practical clinical application of these various theories of progression have remained elusive. We proposed that having a reduced number of functioning lymphatic vessels and impaired lymph drainage (lymphatic vascular insufficiency) in the colon actually protects against progression of inflammatory colitis to colon cancer. Our primary objective was to determine whether there is a reduced incidence of colon cancer in mice with lymphatic deficiency from genetic knockout of angiopoietin 2 (Ang2) compared to controls. This project just completed an extension year to more fully address project objectives.

Body

SOW Task 1-5 Goals Accomplished Summary:

Task 1: ACURO animal use: Completed and in place

Task 2: Produce CRC mouse model: Completed 10 experimental/control groups to death or sacrifice. Data analysis largely completed. Task 2 required considerably more time and greater number of mice to standardize AOM and DSS dosages/regimens and therefore Tasks 3 and 4 have not yet been fully completed.

Task 3: Tissue analysis and imaging: Collected bloods and colonic tissue at sacrifice, performed gross tissue analysis, detailed is ongoing. Pilot-tested 3 non-invasive imaging approaches.

Task 4: Data analysis: Completed most analysis (see below), further analysis is ongoing

Task 5: Summary and Publications: Submitted and revised/updated Final Progress Report to DOD. Two abstracts presented - one as poster/published online; one presented orally and as poster, student 1st prize awarded, published J Inv Med 2014. Manuscript in preparation.

Task 1: Existing Institutional Animal Care and Use Committee protocol was modified and specific approved for all aspects of this project in conjunction with the DOD ACURO Appendix was obtained. Subsequent renewal application was approved by IACUC on 8/16/12 (until 8/16/2015), and renewal was identical (without any changes) for this project protocol. ACURO notification and approval for renewal was obtained.

Task 2: Before the AOM+DSS inflammatory colitis to colorectal cancer model could be implemented in randomized subsets of angiopoietin-2 (Ang-2) knockout (KO) (-/-), Ang-2 haploinsufficient (+/-), and wildtype control (+/+) mice, multiple preliminary experiments were necessary to recalibrate and standardize both the procarcinogen AOM dosage and the DSS regimen to assure both effectiveness as well as acceptable survival for the ~ 2-month period of study. Initially, pilot groups of wildtype +/+ mice (n=72) were chronically administered dextran sodium sulfate (DSS-H20) in a 3-cycle on-off regimen to produce inflammatory colitis and assess colonic tumor formation in the absence of added AOM. Because of recognized variability in the potency of the DSS lots and high mortality (100%) observed in the 3-cycle regimen at our previously tolerated acute 1 cycle regimen of 3% DSS (4), we performed a series of experiments to test scaled down DSS doses stepwise to 2%, then 1.5% to achieve clinical effect, tolerability, and acceptable mortality in KO mice. At the 1.5% DSS dosage without AOM priming, 20/25 mice survived to sacrifice and exhibited clinical symptoms and signs of inflammatory colitis. None showed gross evidence of tumor “bumps” on the colonic mucosal surface but small microscopic CRCs were noted in 4/20 mice. Single AOM dose was also stepwise reduced from 12 mg/kg (0% survival) to 4 mg/kg to assure long-term survival. In AOM-alone mice neither clinical manifestations of inflammatory colitis nor colonic tumors were noted at the 4mg/kg dose when not followed up by the 3 DSS cycles.

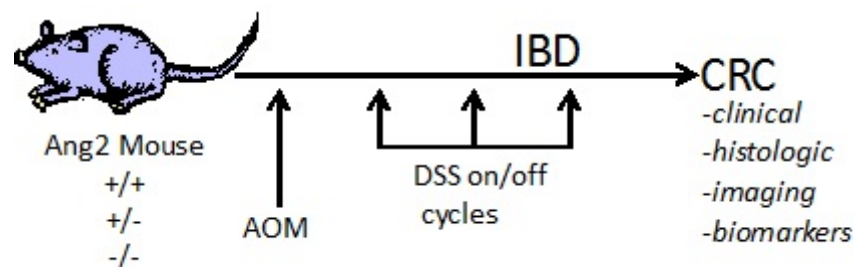


Fig. 1. Using a lymphatic-deficient mouse model (Ang2 -/-) we explored the development of IBD and progression to CRC with clinical monitoring, gross and histologic analysis at sacrifice, non-invasive imaging, and biomarkers.

The experimental model of AOM+ DSS (Fig. 1) was then implemented in successive randomized subsets of Ang-2 +/+, +/- (normal clinical/lymphatic phenotype) and -/- (“lymphatic insufficient”) mice (3) (Table1) (Figs. 2-11). After a single 4 mg/kg procarcinogen AOM dose followed 14 days later by 3 on-off (1 week 1.5% DSS/2 weeks-H20) cycles over ~ 2months, all mouse groups (with similar gender distribution)

showed during their clinical course fluctuating symptomatology of inflammatory colitis (body weight instability, energy level reduction, changes in stool consistency, blood in stool) as observed previously acutely during a single cycle of 3% DSS (4). A similarly high incidence of gross tumors, of variable size and number, protruding into the lumen was observed in all Ang-2 genotypes predominantly in the distal segment of the colon.(Fig. 2) Microscopic examination on H&E-stained sections indicated that these tumors were all non-invasive CRCs (Fig. 2)

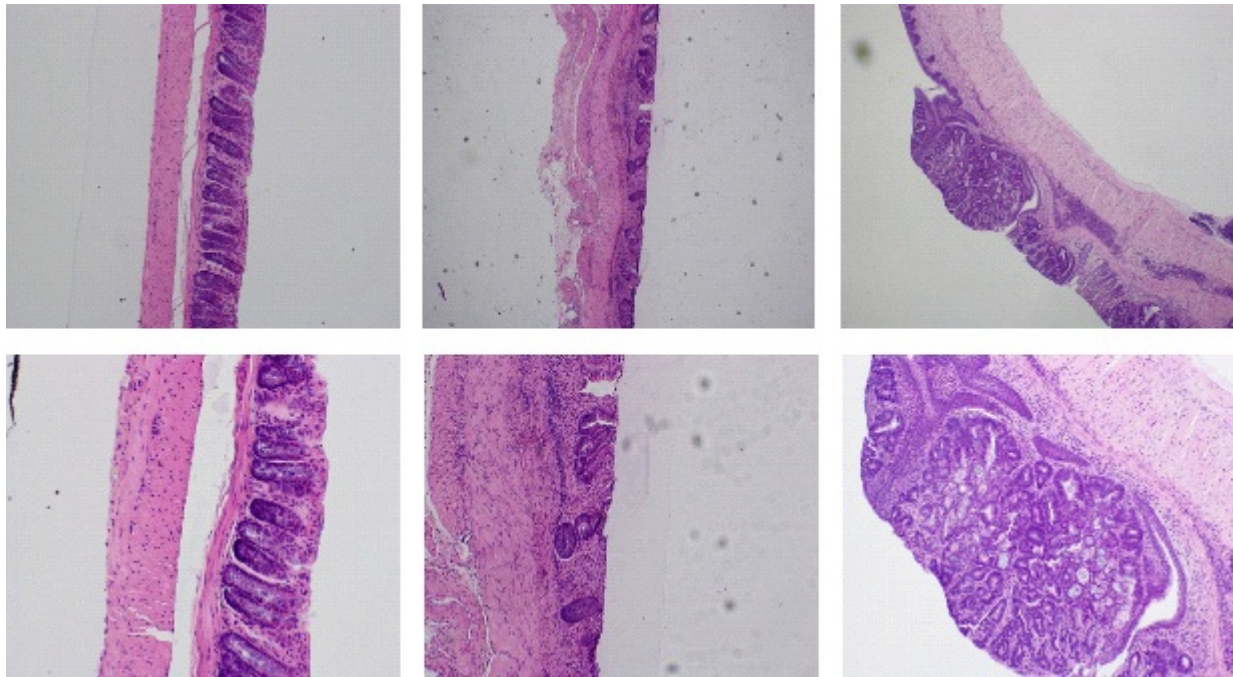


Fig. 2. H&E sections from wildtype (+/+) mice which had undergone no treatment (control, left column) demonstrating normal colon appearance including glandular structures, treatment with DSS alone (middle column) demonstrating loss of glandular structure and inflammatory repair processes, and both AOM+DSS treatment (right column) displaying carcinoma in situ. (original magnification: 10x top row, 20x bottom row).

Table1: Data summary of AOM+DSS experiments in Ang2 genotypes

Ang2 genotype	n	m/f	% survival	Weight initial (grams) (X ± SD)	Weight final (grams) (X ± SD)	Weight change (grams) (X ± SD)	% weight change (X ± SD)	colon length (cm) (X ± SD)	colon weight (grams) (X ± SD)	% CRC incidence	Tumor Burden Score (X ± SD)	ImageJ %Tumor Area (X ± SD)
wt (+/+)	28	17/11	100%	26.8	29.2	+2.411	+0.080	6.668	0.214	89.2%	1.76	5.67%
				5.1	8.3	4.604	0.173	1.196	0.0536		0.879	0.0427
het (+/-)	22	15/7	90.9%	26.9	27.7	+0.075	+0.008	6.337	0.212	95.0%	1.69	4.60%
				7.2	10	6.114	0.214	1.01	0.0503		0.793	0.463
ko (-/-)	31	20/11	51.6%	25.3	24.8	-0.783	-0.021	5.606	0.244	93.4%	2.14	13.50%
				3.4	11.9	4.313	0.146	0.957	0.0538		0.663	0.0378

Compared to Ang-2 +/+, Ang-2 -/- exhibited markedly reduced survival (51.6%) ($p < 0.001$, also c.f. +/-, $p < 0.001$) (Fig. 3), lower final ($p < 0.024$) but similar initial body weight, negative weight change (Fig. 4, $p < 0.014$) (indicator of clinical IBD severity), and shorter colon length (Fig. 5, $p < 0.003$) (indicator of chronic inflammation/fibrosis) but not significantly greater colon weight (Fig. 6). Figures 3-6 utilized two-tailed, unequal variance analysis for t-test and p-value determination (** indicates $p < 0.01$, * indicates $p < 0.05$).

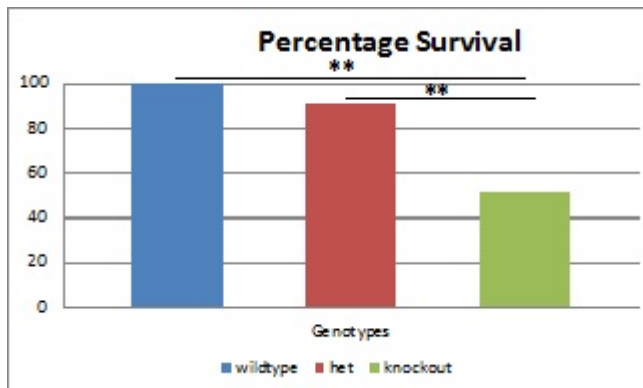


Fig. 3

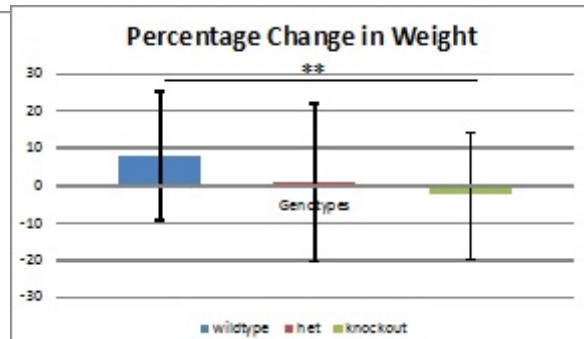


Fig. 4

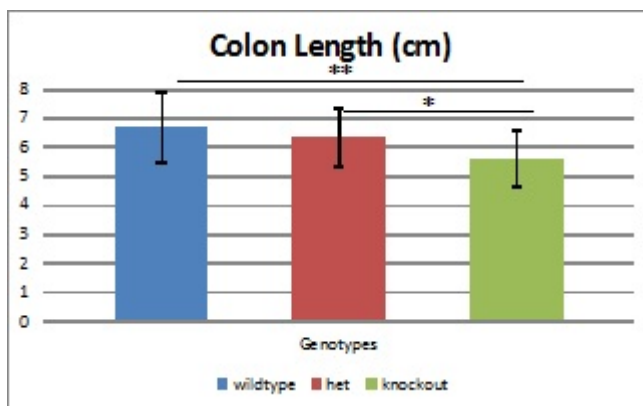


Fig. 5

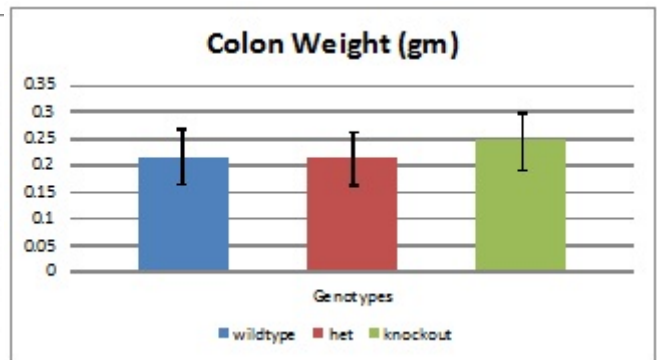


Fig. 6

Although % CRC incidence did not differ among the Ang-2 genotypes (Fig. 7), Ang-2 -/- displayed increased distal colon tumor surface area [Image J analysis (Fig. 8, $p < 0.008$)] and an approaching significantly increased tumor burden "score" (Fig. 9, $p = 0.088$) involved with gross tumor "bumps" protruding into the lumen and some of the largest tumor burdens. Generally, +/- resembled +/+ or showed intermediate findings between +/+ and -/-. Figures 7 and 9 utilized two-tailed, unequal variance analysis for t-test and

p-value determination and Figure 8 utilized non-parametric Mann-Whitney Monte Carlo analysis (** indicates $p < 0.01$).

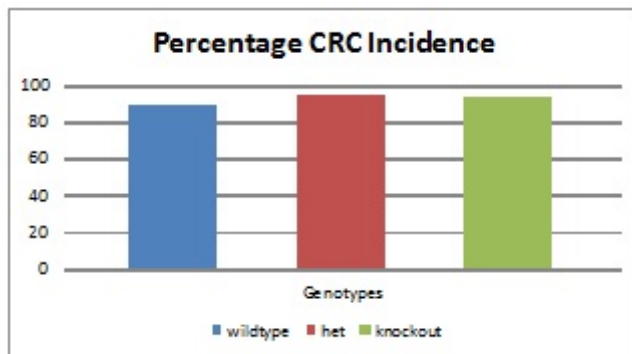


Fig. 7

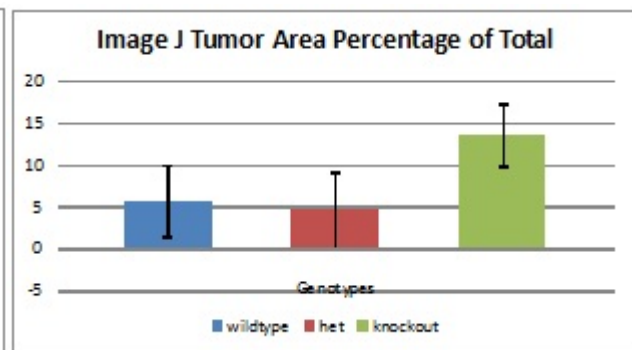


Fig. 8

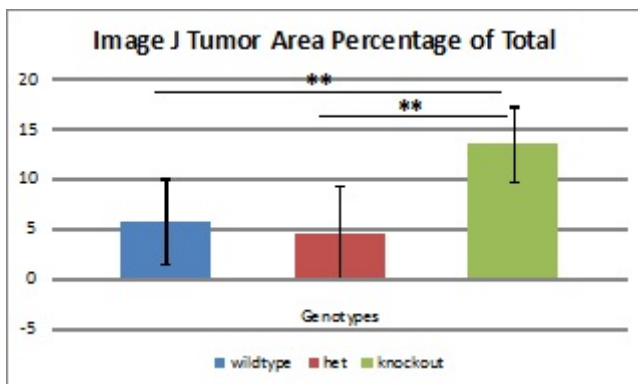


Fig. 9

Further, there was a negative correlation between colon length (~IBD severity) and “tumor burden score” (0-3, none to many distal colon tumor bumps) (quasi R^2 was for Cox and Snell 39% and for Nagelkerke 42.6%, Chi-sq.=13.841, $p < 0.001$) (Fig. 10) and Image J quantitation of distal colon tumor surface area ($R^2=0.321$, $F= 8.974$, coefficient= -3.51, $p=0.007$) (Fig.11), and the small percentage of AOM+DSS mice that did not develop CRC exhibited less colon shortening (histologic inflammatory indices pending).

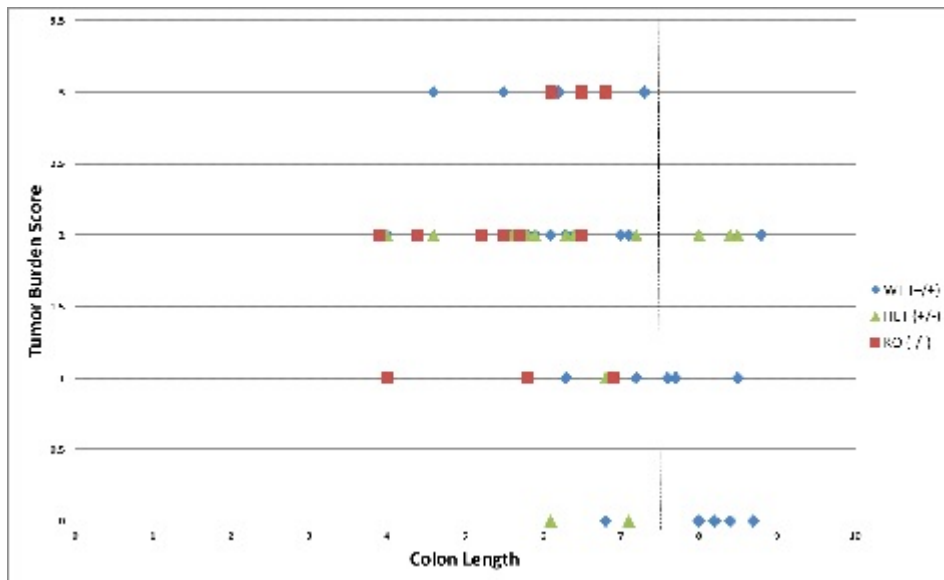


Fig. 10. Tumor burden score vs. colon length for Ang2 genotypes. Vertical bar indicates untreated control mean colon length (see text).

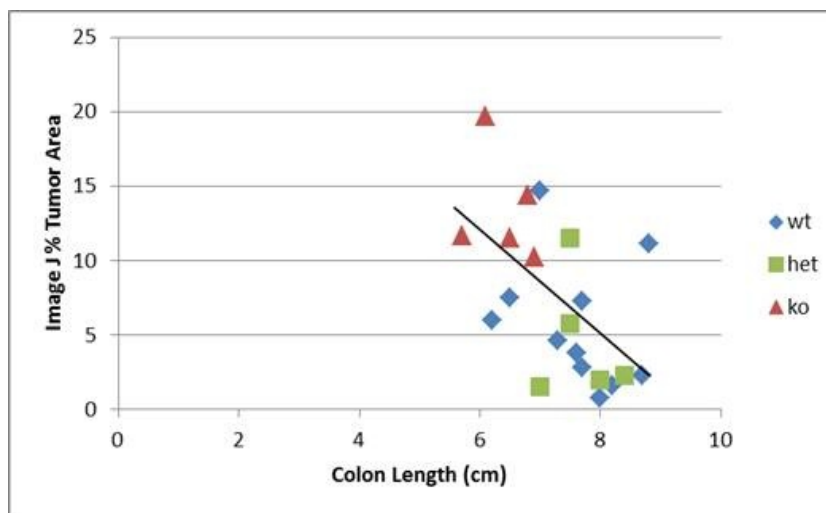


Fig. 11. ImageJ percent distal colon tumor area for all Ang2 genotypes combined was inversely proportional to colon length (see text).

Task 3: Detailed tissue and imaging analysis of the neoplastic changes as well as serial blood biomarker analysis for the completed experimental and control groups is underway (see Task 2) but not yet complete. Gross and dissecting microscopic examination has been carried out on the distal colon segment, and all of the predetermined 4 colon segments identified spanning from the proximal to distal colon, preserved and processed appropriately for further histologic (beyond confirmation of CRC in the distal colon segment) and immunohistochemical examination.

Blood collection at sacrifice has been completed for all mouse groups and experiments, and sera prepared and frozen for batch analysis of tumor/angiogenesis biomarkers.

Instead of a single ELISA assay as originally planned, Ang-2 serum levels will be measured in a specially customized Milliplex MAP Mouse Angiogenesis/Growth Factor Magnetic Bead panel kit just shipped. The panel also includes IL-1B, IL-6, TNF-alpha, and VEGF-C, thereby providing an opportunity to examine inflammatory as well as hemangiogenesis/ lymphangiogenesis biomarker serum levels in experimental and control groups.

The potential of advanced non-invasive, dynamic ultrasound imaging has been explored in individual +/+ mice using the Vevo ultrasound machine, and challenging technical issues with colonic ultrasound imaging have been identified, specifically, tissue depth and gas in the colon. Preliminary success was obtained with UA Medical Imaging collaborator Zhonglin Liu, PhD. Utilization of the novel peptide TCP-1 (recognizes only endothelium of orthotopic colon tumors) with both fluorescent and radioactive markers was attempted in mice with tumors. Confocal imaging of removed distal colon with fluorescent TCP-1 demonstrated focal localization of the tracer to tumor appearing area. (Fig. 12) Non-invasive imaging with ⁹⁹Tc labeled TCP-1 using both the FASTSPECT II and MODPET imaging platforms followed by autoradiography of the removed colon were of interest, but this method is not yet confirmed and will need substantially more experimentation.

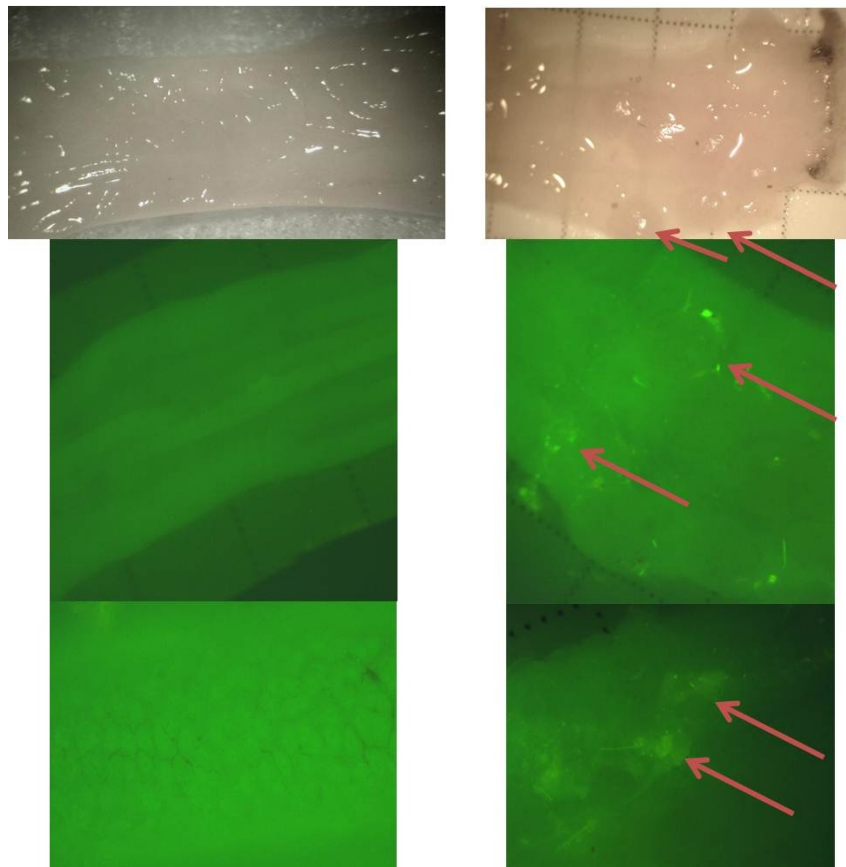


Fig. 12. TCP-1 peptide CRC tumor marker linked with FITC. Normal control wildtype mouse (left) exhibited no obvious tumors on the gross distal colon specimen (top) and in sections using confocal microscopy (middle and bottom). AOM+DSS-treated wildtype mouse (right) demonstrates raised tumor bumps on the gross specimen (arrows, top) and highlighted areas under confocal microscopy (arrows, middle and bottom).

Task 4: Project data analysis has been completed for most components of the project (see Body text, Table 1 and Figs. 2-12 above), 2 abstracts prepared for presentation/publication, but still in process for others and full manuscript as data continue to be collected.

Key Research Accomplishments

- Completed and secured approval of ACURO Appendix
- Revised dextran sodium sulfate (DSS) dosing in our model
- Completed mouse cohorts with chronic exposure to DSS
- Revised dosing and added pro-carcinogen azoxymethane (AOM) to the chronic IBD protocol
- Completed AOM+DSS experimental/control groups and answered Concept grant central question by documenting that Lymphatic Insufficiency/Ang-2 deficiency did not protect against progression of IBD to cancer (CRC incidence and size was not reduced). Instead, both IBD severity and CRC burden were actually increased. Further, CRC tumor burden across all genotypes was inversely proportional to colon length (shortened colon is an indicator of IBD severity).
- In process of completing tissue analyses, Ang-2/IL-1B/ IL-6/TNF-alpha/VEGFC serum levels, and evaluating colonic imaging studies with new modalities.
- Final experimental data analysis is ongoing
- Prepared 2 abstracts on major findings (received 1 prize) and currently preparing full manuscript for presentation and publication

Reportable Outcomes

Work in this project has now largely completed research objectives and the experimental/control groups.

- Washington, J, Rodriguez A, Daley S, Bernas M, Thorn J, Alexander S, Witte M. Inflammatory bowel disease, lymphatic insufficiency, and progression to colorectal cancer: experimental model development and pilot study. Abstract and Presentation at the 5th International Symposium on Cancer Metastasis and the Lymphovascular System: Basis for Rational Therapy and the 8th International Sentinel Node Society (ISNS) Meeting, Monday, May 27 – Wednesday, May 29, 2013 in San Francisco, California. Online Abstract #125 (<http://www.sn-cancermets.org/docs/caa.pdf>) (full abstract attached below)
- Washington, J, Daley S, Rodriguez A, Bernas M, Thorn J, Alexander S, Witte M. Inflammatory colitis, lymphatic insufficiency, and progression to colorectal cancer in an experimental mouse model. Abstract and oral and poster presentation at American Federation for Medical Research, Western Regional Meeting. Carmel,

CA. Jan 23-25, 2014. Journal of Investigative Medicine 62:206, 2014. Student first prize in the Gastroenterology session at the Western Federation for Medical Research meeting in Carmel, California 1/23/14 reporting the project's major findings up until that time. (full abstract attached below)

Conclusion

This project answered the key question of the Concept grant - does Ang-2 deficiency (and "lymphatic insufficiency") protect against IBD progression and CRC development? The answer is clearly No in our experimental model. We have just completed a no-cost extension year, and all experiments have been completed with final laboratory analyses of preserved specimens still in process. Encouraging results in the use of the model and determination of functional model parameters have been obtained for which data analysis is still ongoing to determine significance of some findings and for full reporting. Two abstracts have been completed for meeting presentation 2013-2014, and further reportable outcomes and a full manuscript (1 or more) are anticipated during the coming year.

Final conclusions at this time:

This refined mouse model of IBD/colitis progression to colorectal cancer (CRC) is rapid, reproducible and well-tolerated with high CRC incidence demonstrating that even subacute colitis in the presence of low-dose procarcinogen (tolerated by the normal colon and non-carcinogenic) is a CRC forerunner. Further, lymphatic deficiency, defective lymphangiogenesis, and impaired lymphatic-generated inflammation do not protect against either IBD/colitis clinical severity or its progression to non-invasive CRC (incidence and size not reduced). Whether lymphatic insufficiency/Ang2 deficiency might protect against subsequent CRC invasion and spread/metastasis is unanswered along with an explanation for the apparent worsened IBD and larger size of the primary tumor in Ang2 deficient mice.

References

These references are supplied as background for the project (1,2,6) and the chronic DSS mouse model (5) with our modification to use the Angiopoietin2 knockout mice (3,4) with lymphatic deficiency to explore the role of the lymphatic system in IBD.

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- 2) Colombel JF, Watson AJM, Neurath MF: The 10 remaining mysteries of inflammatory bowel disease. Gut 57:429-433, 2008.
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 - 5) Okayasu I, Yamada M, Mikami T, Yoshida T, Kanno J, Ohkusa T: Dysplasia and carcinoma development in a repeated dextran sulfate sodium-induced colitis model. *J Gastroent Hep.* 17:1078-1083, 2002.
 - 6) Yoffey JM, Courtice, FC (eds): *Lymphatics, Lymph and the Lymphomyeloid Complex*. Academic Press, London, 942 p, 1970.

Appendix: Abstracts Attached Below

5th International Symposium on Cancer Metastasis and the Lymphovascular System: Basis for Rational Therapy and the 8th International Sentinel Node Society (ISNS) Meeting, Monday, May 27 – Wednesday, May 29, 2013 in San Francisco, California. Online Abstract #125 (<http://www.sn-cancermets.org/docs/caa.pdf>)

Inflammatory bowel disease, lymphatic insufficiency, and progression to colorectal cancer: experimental model development and pilot study.

Washington, J¹, Rodriguez A¹, Daley S¹, Bernas M¹, Thorn J², Alexander S³, Witte M¹. Departments of Surgery¹ and Pathology², University of Arizona, Tucson, AZ, Department of Molecular and Cellular Physiology³, Louisiana State University, Shreveport, LA.

Background: Inflammatory bowel disease (IBD) is a well-recognized risk factor for colorectal cancer (CRC) [~15-20% lifetime risk in ulcerative colitis (UC)] but the underlying mechanisms are not well understood. The lymphatic system has been implicated in both IBD pathogenesis and pathophysiology as well as in CRC growth and spread. Previously, we showed that in experimental acute dextran sodium sulfate (DSS) colitis, mice with severe lymphatic deficiency from knockout of vascular remodeling factor angiopoietin-2 gene (Ang2^{-/-}) exhibited marked reduction in lymphangiogenesis (but not hemangiogenesis) as well as downregulation of inflammatory markers. This study aimed to extend these observations to a chronic UC model and further, to examine progression to CRC.

Materials and Methods: C57B6 adult mice (Ang2 ^{+/+}, ^{+/-}, ^{-/-}) (Regeneron Pharmaceuticals, Inc) were divided into 4 groups: Group 1 received a single 4 or 12 mg/kg dose of procarcinogen azoxymethane (AOM) intraperitoneally, and 14 days later, 1-1/2% DSS in drinking water for 7 days followed by 14 days off DSS, and the on-off DSS cycle repeated once or twice depending on clinical severity score. Control mice included AOM alone (Group2), DSS cycles without AOM (Group 3), or sham untreated (Group 4). Clinical severity scores incorporating changes in body weight, energy level, and stool consistency and occult blood were followed serially. At sacrifice, colon length was measured and tissue samples obtained in 4 segments from proximal to distal colorectum and assessed by an inflammatory disease index and for tumor size and histologic features.

Results: Groups 1 and 3 exhibited similar clinical severity scores and mortality with reduced survival (46.5% c.f. 90%) in Ang2^{-/-} whereas Groups 2 (4mg/kg) and 4 were unaffected. Group 2 exhibited rapid 100% mortality at 12 mg/kg but 0% at 4mg/kg AOM dose. Group 1 had a 91% tumor incidence in the distal segment, and in the few mice without gross CRC, clinical UC severity score tended to be lower. Ang 2^{-/-} were not protected from CRC (some of the largest tumor burdens), and clinical severity scores were not improved and indeed, tended to be worse.

Conclusions/Discussion: Thus, this refined mouse model of CRC progression in UC/IBD is rapid, reproducible, and well-tolerated with high incidence of gross CRC, demonstrating that even subacute IBD in the presence of a procarcinogen at low dose tolerated by the normal colon is a forerunner of CRC. Further, lymphatic deficiency, defective lymphangiogenesis, and impairments in lymphatic-generated inflammation do not appear to protect against either the clinical severity of IBD or its progression to CRC.

Abstract for presentation at American Federation for Medical Research, Western Regional Meeting. Carmel, CA. Jan 23-25, 2014
Journal of Investigative Medicine (in press)

INFLAMMATORY COLITIS, LYMPHATIC INSUFFICIENCY, AND PROGRESSION TO COLORECTAL CANCER IN AN EXPERIMENTAL MOUSE MODEL.

Washington, J¹, Daley S¹, Rodriguez A¹, Bernas M¹, Thorn J², Alexander S³, Witte M¹.
Departments of Surgery¹ and Pathology², University of Arizona, Tucson, AZ, Department of Molecular and Cellular Physiology³, Louisiana State University, Shreveport, LA.

Purpose of Study: Inflammatory bowel disease (IBD) is a well-recognized risk factor for colorectal cancer (CRC) [~15-20% lifetime risk in ulcerative colitis (UC)]. The lymphatic system has been implicated in both IBD pathophysiology and CRC growth/spread. Previously, we showed in acute dextran sodium sulfate (DSS) colitis, lymphatic deficient mice [knockout of angiopoietin-2 (Ang2)] exhibited reduced lymphangiogenesis and down-regulated inflammatory markers. This study extends these observations to chronic UC and examines progression to CRC.

Methods Used: C57B6 adult mice (Ang2 +/+, +/-, -/-) (Regeneron) were divided into 4 groups: Group 1 -single 4 or 12 mg/kg IP dose of procarcinogen azoxymethane (AOM) and 14 days later, 1-1/2% DSS in drinking water for 7 days then 14 days off DSS (cycle repeated 1-2X ~clinical severity score). Controls included AOM alone (Group2), DSS cycles without AOM (Group 3), or untreated (Group 4). Clinical severity scores (changes in body weight, energy, stool consistency, occult blood) were followed. At sacrifice, colon length was measured and tissue sampled in 4 segments from proximal to distal colorectum and assessed by inflammatory index, tumor burden, and histologic features.

Summary of Results: Groups 1 and 3 showed similar clinical severity and mortality and reduced survival (46.5% c.f. 90%) in Ang2-/- whereas Groups 2 (4mg/kg AOM) and 4 were unaffected. Group 2 exhibited rapid 100% mortality at 12 mg/kg AOM but 0% at 4mg/kg. Group 1 had 91% non-invasive CRC incidence in the distal segment. Ang2-/- were not protected from CRC, and clinical severity tended to be worse. Tumor burden in -/- mice (13.5% of distal colon surface) was significantly higher than +/- (4.6%, p=0.008) and +/+ (5.5%, p=0.007)(n.s. +/- vs. +/+).

Conclusions: This refined mouse model of UC progression to CRC is rapid, reproducible, and well-tolerated with high CRC incidence demonstrating that even subacute UC in the presence of a low-dose procarcinogen tolerated by the normal colon is a CRC forerunner. Further, lymphatic deficiency, defective lymphangiogenesis, and impaired lymphatic-generated inflammation do not protect against either the UC clinical severity or its progression to non-invasive CRC.